PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 15/28, C12P 21/06 C07H 15/00, A01N 43/04	**A1**	(11) International Publication Number: **WO 93/11162** (43) International Publication Date: 10 June 1993 (10.06.93)

(21) International Application Number: PCT/US92/10140

(22) International Filing Date: 25 November 1992 (25.11.92)

(30) Priority data:
07/801,798   29 November 1991 (29.11.91) US

(71) Applicant: PROTEIN DESIGN LABS, INC. [US/US]; 2375 Garcia Avenue, Mountain View, CA 94043 (US).

(72) Inventors: TSO, J., Yun ; 445 Oak Grove Avenue, #16, Menlo Park, CA 94025 (US). KOSTELNY, Sheri, A. ; 310 Chiquita Avenue, Mountain View, CA 94041 (US). COLE, Michael, S. ; 990 College Avenue, Palo Alto, CA 94306 (US).

(74) Agents: DUNN, Tracy, J. et al.; Townsend and Townsend, One Market Plaza, 20th Fl. Steuart Tower, San Francisco, CA 94105 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: BISPECIFIC ANTIBODY HETERODIMERS

(57) Abstract

Methods for producing and using bispecific antibodies formed by leucine zippers are provided. Leucine zippers capable of preferentially forming heterodimers are respectively linked to epitope binding components comprising different binding specificities. Bispecific antibodies are formed by pairwise association of the leucine zippers, forming a heterodimer which links the two distinct epitope binding components. Heterodimerization can occur by interaction of the two leucine zipper regions, forming a bispecific antibody. Such a bispecific antibody may be further stabilized by the formation of intermolecular chemical bonds, such as disulfide bonds, between the two monomeric subunits. Subsequent to the formation of such intermolecular bonds between the monomeric subunits, the leucine zippers may be removed or retained. Bispecific antibodies produced by these methods are substantially pure and may be produced in high yields and on a large scale. Alternatively, bifunctional heterodimers may be formed by linking an epitope binding component to a macromolecular species that is not an epitope binding component.

BISPECIFIC ANTIBODY HETERODIMERS

TECHNICAL FIELD OF THE INVENTION

The invention relates to bispecific antibodies, epitope binding components capable of forming specific heterodimers with other epitope binding components, methods for producing such bispecific antibodies and epitope binding components, methods for using such bispecific antibodies and epitope binding components, and pharmaceutical compositions containing such bispecific antibodies and epitope binding components.

BACKGROUND OF THE INVENTION

Bispecific antibodies are antibodies with dual epitope binding specificities, with one specificity being the capacity to bind a first epitope and a second specificity being the capacity to bind a second epitope.

Such bispecific antibodies are, in some embodiments, potentially valuable molecules for immunotherapy. For example, bispecific antibodies can crosslink cytotoxic effector cells to target cells (Segal and Snider, (1989) _Chem. Immunol._ 47:179), resulting in the killing of the target cell.

Although numerous bispecific antibodies have been shown effective _in vitro_ (Gililand et al., (1988) _Proc. Natl. Acad. Sci. USA_ 85:7719; Lanzavecchia and Scheidegger, (1987) _Eur. J. Immunol._ 17:105; Stearz and Bevan, (1986) _Immunol. Today_ 7:241; Berg et al., (1991) _Proc. Natl. Acad. Sci. USA_ 88:4732), few have been tested clinically as therapeutic agents. One of the reasons for the slow development of bispecific antibodies as therapeutic agents has been the difficulty in manufacturing them in sufficient purity and quantity.

Bispecific antibodies have been produced by chemical cross-linking, by hybrid-hybridomas (Milstein and Cuello, (1984) _Immunol. Today_ 5:299) or transfectomas, or by disulfide

exchange at the hinge of two different Fab'. The first method
yields heterogeneous and ill-defined products. The second
method requires extensive purification of the bispecific
antibodies from many hybrid-antibody side products, the
5 presence of which may interfere with the cell cross-linking
activity. The disulfide exchange method applies essentially
only to F(ab')$_2$, and is thus limited by the susceptibility of
the monoclonal antibodies to cleavage by enzyme digestion
(Parham, (1983) J. Immunol. 131:2895). Further, since Fab'
10 have little affinity for each other, very high protein
concentrations are required for the formation of the inter-Fab'
disulfide bonds. The disulfide exchange method has been
improved by the use of Ellman's reagent to modify one of the
Fab' prior to oxidation with the other Fab', reducing the
15 incidence of homodimerization (Brennan et al., (1985) Science
229:81). However, even with this improvement, heterodimeric
F(ab')$_2$ can rarely be produced in better than 50% yield
(Glennie et al., (1987) J. Immunol. 139:2367).

Thus, there remains a significant need for improved
20 methods for efficiently producing bispecific antibodies and
other similar compounds at high purity.

SUMMARY OF THE INVENTION

The present invention includes novel methods for the
25 production of bispecific antibodies that include: 1) producing
F(ab')$_2$ and/or other epitope binding components directly, e.g.,
by gene expression, and 2) utilizing heterodimer-forming
sequences to ensure efficient production of the bispecific
antibodies. The sequences employed may be derived from the
30 leucine zipper (Landschulz et al., (1988) Science 240:1759, and
for review, see Maniatis and Abel, (1989) Nature 341:24, both
of which are incorporated herein by reference) regions of the
transcription factors Fos and Jun. Leucine zippers are
specific amino acid sequences about 20-40 residues long with
35 leucine typically occurring at every seventh residue. Such
zipper sequences form amphipathic α-helices, with the leucine
residues lined up on the hydrophobic side for dimer formation.
Peptides corresponding to the leucine zippers of the Fos and

Jun proteins form heterodimers preferentially (O'Shea et al.
(1989) <u>Science</u> <u>245</u>:646).

In the present invention, two leucine zipper
sequences are employed to promote bispecific $F(ab')_2$ formation
5   when fused to two different Fab'. Bispecific antibodies are
produced by the methods of the invention, whereby the pairwise
association of two distinct zipper sequences links a first Fab'
or other epitope binding component containing one zipper to a
second Fab' or other epitope binding component that contains
10 the other zipper. In these embodiments, a heterodimeric
molecule will comprise the binding properties of both epitope
binding components.

The present invention provides bispecific antibodies
formed from a first protein which includes a leucine zipper
15 linked to an epitope binding component and a second protein
which includes a leucine zipper linked to an epitope binding
component, wherein the leucine zippers of the first and second
proteins have pairwise affinity so that heterodimers comprising
the first and second proteins are formed. In some embodiments
20 of the invention, one of the leucine zippers is a Fos leucine
zipper or a Jun leucine zipper. In one embodiment of the
invention, one leucine zipper is a Fos leucine zipper and the
other leucine zipper is a Jun leucine zipper. The invention
also provides bispecific antibodies which have a first and
25 second leucine zipper of the structural sequence (leucine-X_1-
X_2-X_3-X_4-X_5-X_6)$_n$, wherein each of X_1, X_2, X_3, X_4, X_5, and X_6 is
one of the conventional 20 amino acids and n is an integer that
is at least 3.

The present invention provides bispecific antibodies
30 which bind to a human IL-2 receptor and bispecific antibodies
which bind to a human CD3 protein. The present invention also
provides bispecific antibodies that have at least one epitope
binding component that is an Fab'. Some embodiments of the
present invention have at least one epitope binding component
35 that is a humanized immunoglobulin.

The present invention also provides methods for
preparing bispecific antibodies where a first and a second
protein are produced, each including an epitope binding

4

component and a leucine zipper, and the first and second proteins are contacted under conditions that permit heterodimer formation to form a bispecific antibody. The contacting of the first and second proteins may occur _in vitro_ or _in vivo_ in a
5 single cell that expresses both proteins. In some embodiments, the methods employ proteins that have a Fos or Jun leucine zipper or other similar leucine zippers corresponding to the formula (leucine-X_1-X_2-X_3-X_4-X_5-X_6)$_n$, wherein each of X_1, X_2, X_3, X_4, X_5, and X_6 is one of the conventional 20 amino acids and n
10 is an integer that is at least 3. In some methods of the invention, the F(ab'-zipper)$_2$ heterodimer bispecific antibody may be produced as a final product, or it may be cleaved (e.g., cleavage of an asparagine-glycine peptide bond by hydroxylamine) to remove the leucine zippers to yield a F(ab')$_2$
15 bispecific antibody wherein the two Fab' are chemically linked (e.g., by a disulfide bond). Epitope binding components other than Fab' may be used in methods of the present invention to form bispecific antibodies which are linked by a chemical bond but which have had the leucine zippers removed.
20   The present invention also provides polynucleotides that encode proteins that include an epitope binding component and a leucine zipper, particularly including an epitope binding component that has the V, C_{H1}, and hinge domains of an antibody heavy chain, and more particularly a Fab'.
25   Other bispecific antibodies, wherein epitope binding components other than Fab' are used, and/or wherein one component of the bispecific antibody is a macromolecular species that is not an epitope binding component, can also be generated by these methods.
30   The present invention also encompasses pharmaceutical compositions of bispecific antibodies, therapeutic uses of such bispecific antibodies, methods and compositions for using bispecific antibodies in diagnostic and research applications.

35

BRIEF DESCRIPTION OF THE DRAWINGS

5

Fig. 1. Sequences of the Jun (A) and Fos (B) leucine zipper. Arrows indicate the splicing sites between intron H:C_H2 and exon C_H2.

Fig. 2. Diagrams of plasmid constructs for expression of anti-Tac-Jun (A) and anti-CD3-Fos (B). Schematic diagrams of the protein products are also shown. Coding sequences are shown as boxes. Symbols for restriction sites are: B, BamHI; F, FspI; H, HindIII; S, SalI; X, XbaI; and Xh, XhoI.

Fig. 3. SDS PAGE analysis of anti-Tac-Jun and anti-CD3-Fos purified by the rat anti-mouse kappa Sepharose. Proteins were analyzed on a 12.5% polyacrylamide gel and stained with Coomassie blue. Lanes 1, 2, 3 are purified anti-CD3-Fos, and lanes 4, 5, 6 anti-Tac-Jun run under nonreducing conditions. Lanes 7, 8, 9 are anti-CD3-Fos and lanes 10, 11, 12 anti-Tac-Jun run under reducing conditions. M.W markers are: phosphorylase b, 94 kd; bovine serum albumin, 67 kd; ovalbumin, 43 kd; carbonic anhydrase, 30 kd; soy bean trypsin inhibitor, 20 kd; and lysozyme, 14 kd. Abbreviations are: $F(ab')_2$, $F(ab'$-zipper$)_2$; LC, light chain; and Fd, Fd-zipper.

Fig. 4. Fractionation of the spent medium of the anti-Tac-Jun and anti-CD3-Fos expressing supertransfectant on BAKERBOND ABx column by FPLC. (A) Absorbance profile of proteins at 280 nm as they were eluted by a gradient of $(NH_4)_2SO_4$. Proteins that were eluted early (fraction T) are mostly medium supplements such as transferrin and insulin. (B) Mouse IgG positive fractions as determined by ELISA. Absorbance at 414 nm represents the color developed by the secondary antibodies, which were peroxidase-conjugated goat anti-mouse IgG. (C) Anti-CD3 (O) and anti-Tac (■) activities for different fractions assayed by flow cytometry.

Fig. 5. Targeted cytotoxicity mediated by bispecific antibody from fraction II. Effectors and ^{51}Cr-labeled target cells at ratios of 100:1 (O), 25:1 (O), and 10:1 (■) were incubated with various dilutions of fraction II (Fig. 4A) for specific lysis. Points represent means of triplicate determinations. Protein concentrations were estimated by flow cytometry.

6

Fig. 6. Formation of bispecific F(ab'-zipper)$_2$ *in vitro* as analyzed by SDS PAGE under nonreducing conditions. Lane 1, anti-Tac-Jun prior to reduction; lane 3, anti-Tac-Jun after reduction with 4 mM 2-mercaptoethylamine; lane 4, anti-CD3-Fos after reduction with 2 mM 2-mercaptoethylamine and lane 5, anti-Tac-Jun x anti-CD3-Fos after dialysis against redox buffer. All protein samples were treated with 20 mM of iodoacetamide to block free sulfhydryls before boiling in sample SDS buffer. M.W. markers are the same as those used in Fig. 3. They were boiled in reducing sample SDS buffer before use. Abbreviations are: F(ab')$_2$, F(ab-zipper)$_2$; Fab', Fab'-zipper; and LC, light chain.

Fig. 7. Fractionation of the bispecific F(ab-zipper)$_2$ formed *in vitro*. Anti-Tac-Jun x anti-CD3-Fos formed *in vitro* was dialyzed against 10 mM MES buffer, pH 5.2 before loading onto a BAKERBOND ABx column. Proteins that were bound to the column were eluted by a gradient of $(NH_4)_2SO_4$. (A) Absorbance profile at 280 nm. (B) Anti-CD3 (O) and anti-Tac (■) activity for different fractions assayed by flow cytometry.

Fig. 8. SDS PAGE analyses of the peak fractions in Fig. 7 run under (A) nonreducing or (B) reducing conditions. Lane 1, the ABx column flow-through fraction; lane 2, fraction I; lane 3, fraction II; lane 4, fraction III; and lane 5, fraction IV. More volume (5-fold) was taken from fraction II and fraction IV for sampling. M.W. markers are the same as those used in Fig. 3. Abbreviations are: F(ab')$_2$ F(ab-zipper)$_2$; LC, light chain; and Fd, Fd-zipper.

Fig. 9. Targeted cytotoxicity mediated by bispecific anti-Tac-Jun x anti-CD3-Fos formed *in vitro*. Effectors and ^{51}Cr-labeled target cells at a ratio of 25:1(O) and 10:1 (O) were incubated with various concentrations of fraction III (Fig. 7A) for specific lysis.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, bispecific antibodies, methods to produce such bispecific antibodies, pharmaceutical compositions of bispecific antibodies, therapeutic uses of such bispecific antibodies, and methods and

7

compositions for using bispecific antibodies in diagnostic and research applications are provided.

Definitions

5   "F(ab')$_2$ heterodimer" is defined herein as a dimer comprising a first Fab' having a binding specificity for a first epitope, and a second Fab' having a binding specificity for a second epitope, wherein the first and second epitopes are nonidentical.

10   "Fab'-zipper" is defined as an Fab' linked to a leucine zipper.

     "F(ab'-zipper)$_2$ heterodimer" is defined herein as a dimer comprising a first Fab' having a binding specificity for a first epitope and linked to a leucine zipper, and a second

15 Fab' having a binding specificity for a second epitope and linked to a leucine zipper, wherein said first and second epitopes are nonidentical.

     "Epitope binding components" of the present invention refer to proteins consisting of one or more polypeptides

20 substantially encoded by genes of the immunoglobulin superfamily (e.g., see The Immunoglobulin Gene Superfamily, A.F. Williams and A.N. Barclay, in Immunoglobulin Genes, T. Honjo, F.W. Alt, and T.H. Rabbitts, eds., (1989) Academic Press: San Diego, CA, pp.361-387, which is incorporated herein

25 by reference). For example, but not for limitation, an epitope binding component may comprise part or all of a heavy chain and part or all of a light chain, or may comprise only part or all of a heavy chain. However, an epitope binding component must contain a sufficient portion of at an immunoglobulin

30 superfamily gene product so as to retain the property of binding to a specific target, or epitope.

Leucine zipper

     Recently, a protein structural motif designated as a

35 "leucine zipper" has been identified (Landschulz et al., (1988) Science 240:1759). The leucine zipper has been defined in the art as a stretch of about 35 amino acids containing 4-5 leucine residues separated from each other by six amino acids (Maniatis

and Abel, (1989) <u>Nature</u> <u>341</u>:24). The leucine zipper has been
found to occur in a variety of eukaryotic DNA-binding proteins,
such as GCN4, C/EBP, c-fos gene product (Fos), c-jun gene
product (Jun), and c-myc gene product. In these proteins, the
5 leucine zipper creates a dimerization interface wherein
proteins containing leucine zippers may form stable homodimers
and/or heterodimers.

    Molecular analysis of the protein products encoded by
two proto-oncogenes, c-fos and c-jun, has revealed such a case
10 of preferential heterodimer formation. Both of these DNA-
binding proteins contain leucine zipper regions, however while
Jun is capable of forming a homodimer, and Fos and Jun are
capable of heterodimerizing with each other, there has been
little evidence for the homodimerization of Fos (Gentz et al.,
15 (1989) <u>Science</u> <u>243</u>:1695; Nakabeppu et al., (1988) <u>Cell</u> <u>55</u>:907;
Cohen et al., (1989) <u>Genes Dev.</u> <u>3</u>:173). Thus, the Fos leucine
zipper is able to preferentially dimerize with Jun because of a
characteristic interaction at the helix interface between the
Jun leucine zipper and the Fos leucine zipper (O'Shea et al.
20 <u>op.cit.</u>; Schuemann et al., (1991) <u>Nucleic Acids Res.</u> <u>19</u>:739).

    Synthetic peptides comprising the leucine zipper
regions of Fos and Jun are sufficient by themselves to mediate
heterodimer formation, and, where the amino-termini of the
synthetic peptides each include a cysteine residue to permit
25 intermolecular disulfide bonding, heterodimer formation occurs
to the substantial exclusion of homodimerization.

    Leucine zippers of the present invention have the
general structural formula known as the heptad repeat (Leucine-
$X_1-X_2-X_3-X_4-X_5-X_6)_n$, where X may be any of the conventional 20
30 amino acids (<u>Proteins, Structures and Molecular Principles</u>,
(1984) Creighton (ed.), W.H. Freeman and Company, New York,
which is incorporated herein by reference), but are most likely
to be amino acids with high α-helix forming potential, for
example, alanine, valine, aspartic acid, glutamic acid, and
35 lysine (Richardson and Richardson, (1988) <u>Science</u> <u>240</u>:1648),
and n may be 3 or greater, although typically n is 4 or 5. The
20 conventional amino acids are: glycine, proline, lysine,
arginine, histidine, methionine, tryptophan, phenylalanine,

9

isoleucine, leucine, valine, alanine, serine, threonine,
cysteine, glutamine, asparagine, tyrosine, aspartic acid, and
glutamic acid.

5   The leucine zippers of the present invention have
pairwise affinity. Leucine zippers form amphipathic alpha
helices, and more specifically form coiled coils. Pairwise
affinity is defined as the capacity for one specie of leucine
zipper, for example but not for limitation, the Fos leucine
zipper, to predominantly form heterodimers with another specie
10   of leucine zipper, for example but not for limitation, the Jun
leucine zipper, such that heterodimer formation is preferred
over homodimer formation when two species of leucine zipper are
present in sufficient concentrations. Thus, predominant
formation of heterodimers leads to a dimer population that is
15   typically 50 to 75 percent, preferentially 75 to 85 percent,
and most preferably more than 85 percent heterodimers. "Fos
Leucine zipper" is defined as a sequence of amino acids
substantially similar to the sequence shown in Fig. 1(b). "Jun
leucine zipper" is defined as a sequence of amino acids
20   substantially similar to the sequence shown in Fig. 1(a).
Those of skill in the art will understand that leucine zippers
of the present invention may comprise amino acids sequences
that are not identical to those shown in Fig. 1, by, for
example but not for limitation, having internal or terminal
25   additions, deletions, or substitutions, or by having
rearrangement of the order of the heptad repeats. For
illustration, but not for limitation, the invention encompasses
additions or substitutions of terminal amino acids that
comprise glycine and/or cysteine.

30   The leucine zipper regions of the Jun and Fos
proteins normally serve to bind the proteins together to form a
transcription factor, AP-1. The Jun and Fos zippers will also
dimerize other proteins to which they have been genetically
fused, such as two Fab' halves of a bispecific antibody.
35   Because the pairwise association of the two zipper peptides
results in a much greater tendency to form heterodimers than
homodimers, formation of the desired product is enhanced.

Bispecific antibodies

Bispecific antibodies can be formed by joining two epitope binding components that have different binding specificities.

"Epitope binding components" of the present invention refer to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin superfamily genes and having specific binding affinity for an epitope of an antigen. The recognized immunoglobulin gene superfamily is described in The Immunoglobulin Gene Superfamily, A.F. Williams and A.N. Barclay, op. cite). Specific examples of immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies; including, for example, Fv, Fab, and $F(ab)_2$, as well as in single chains (e.g., Huston, et al., Proc. Nat. Acad. Sci. U.S.A., 85:5879-5883 (1988) and Bird, et al., Science, 242:423-426 (1988), which are incorporated herein by reference). (See, generally, Hood, et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323:15-16 (1986), which are incorporated herein by reference). Other examples of epitope binding components include T-cell antigen receptors and the CD4 protein, which binds to an epitope on MHC protein.

It is well known that native forms of "mature" immunoglobulins will vary somewhat in terms of length by deletions, substitutions, insertions or additions of one or more amino acids in the sequences. Thus, both the variable and constant regions are subject to substantial natural modification, yet are "substantially identical" and still capable of retaining their respective activities. Human constant region and rearranged variable region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells. Similar methods can be used to isolate nonhuman immunoglobulin sequences from non-human sources. Suitable source cells for the DNA sequences and host cells for expression and secretion can be obtained from a number of sources, such as the American Type

Culture Collection ("Catalogue of Cell Lines and Hybridomas,"
Fifth edition (1985) Rockville, Maryland, U.S.A., which is
incorporated herein by reference).

    In addition to these naturally-occurring forms of
5   immunoglobulin chains, "substantially identical" modified heavy
and light chains can be readily designed and manufactured
utilizing various recombinant DNA techniques well known to
those skilled in the art. For example, the chains can vary
from the naturally-occurring sequence at the primary structure
10 level by several amino acid substitutions, terminal and
intermediate additions and deletions, and the like.
Alternatively, polypeptide fragments comprising only a portion
of the primary structure may be produced, which fragments
possess one or more immunoglobulin activities (e.g., binding
15 activity). In particular, it is noted that like many genes,
the immunoglobulin-related genes contain separate functional
regions, each having one or more distinct biological
activities. In general, modifications of the genes encoding
the desired epitope binding components may be readily
20 accomplished by a variety of well-known techniques, such as
site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97
(1979) and Roberts, S. et al., Nature 328:731-734 (1987), both
of which are incorporated herein by reference). In preferred
embodiments of the invention, the epitope binding component is
25 encoded by immunoglobulin genes that are "chimeric" or
"humanized" (see, generally, Co and Queen (1991) Nature
351:501, which is incorporated herein by reference).

    Suitable epitope binding components may be produced
by those of skill in the art from DNA sequences or monoclonal
30 antibody sources well-known in the art, and described with more
particularity in WO90/07861 and U.S.S.N. 07/310,252, which are
incorporated herein by reference.

    The Fab'-Jun (Fab' containing a Jun leucine zipper)
and Fab'-Fos (Fab' containing a Fos leucine zipper) proteins
35 can be used to produce bispecific antibodies either in vivo, by
coexpression in one cell line, or by mixing in vitro after
expression in separate cells. The in vitro mixing procedure is
preferred for large-scale production of bispecific antibodies.

In addition to Fab', other epitope binding components may be
linked to a Jun or Fos leucine zipper, and combined by in vitro
mixing.

The in vitro mixing procedure has the advantage that
5   a particular Fab'-Fos or Fab'-Jun need only be generated once,
as it can then be combined with a variety of epitope binding
components that contain the complementary leucine zipper. For
example, the T cell binding component of a bispecific antibody,
an Fab' comprising a Fos leucine zipper and a binding
10   specificity for the T cell antigen CD3, need only be generated
once, as it can then be combined with any one of a variety of
epitope binding components that contain the Jun leucine zipper,
such as an Fab'-Jun molecule that has a binding affinity for a
desired target cell. In addition, Fab fragments have been
15   produced in Esherichia coli at high levels, so that the F(ab'-
zipper)$_2$ bispecific antibodies may also potentially be produced
economically in large quantity, making clinical trials
possible.

Leucine zippers linked to epitope binding components
20   may be produced in various ways. For example but not
limitation, polynucleotide sequences encoding a fusion protein
comprising a leucine zipper may be expressed by a cellular host
or in vitro translation system. Alternatively, leucine zippers
and/or epitope binding components may be produced separately,
25   either by chemical peptide synthesis, by expression of
polynucleotide sequences encoding the desired polypeptides, or
by cleavage from other proteins containing leucine zippers,
antibodies, or macromolecular species, and subsequent
purification. Such purified polypeptides may be linked by
30   peptide bonds, with or without intervening spacer amino acid
sequences, or by non-peptide covalent bonds, with or without
intervening spacer molecules, said spacer molecules being
either amino acids or other non-amino acid chemical structures.
Regardless of the method or type of linkage, such linkage may
35   be reversible. For example but not limitation, such reversible
linkage may comprise a chemically labile bond, either peptidyl
or otherwise, which may be cleaved spontaneously or upon
treatment with heat, electromagnetic radiation, proteases, or

chemical agents. Two examples of such reversible linkage are offered for illustration and not for limitation, they are: (1) a linkage comprising a Asn-Gly peptide bond which can be cleaved by hydroxylamine, and (2) a disulfide bond linkage
5 which can be cleaved by reducing agents.

Generally, the nomenclature used hereafter and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA
10 isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook
15 et al., <u>Molecular Cloning - A Laboratory Manual</u>, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the
20 information contained therein is incorporated herein by reference.

The nucleic acid sequences of the present invention capable of ultimately expressing the desired bispecific antibodies can be formed from a variety of different
25 polynucleotides (genomic or cDNA, RNA, etc.) by a variety of different techniques. Joining appropriate genomic sequences is presently the most common method of production, but cDNA and synthetic sequences may also be utilized (<u>see</u>, European Patent Application Nos. 85102655.8, 85305604.2, 84302368.0 and
30 85115311.4, as well as PCT Application Nos. GB85/00392 and US86/02269, all of which are incorporated herein by reference).

The DNA constructs will typically include an expression control DNA sequence operably linked to the coding sequences, including naturally-associated or heterologous
35 promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the

host is maintained under conditions suitable for high level
expression of the nucleotide sequences, and the collection and
purification of the bispecific antibodies.

As stated previously, the DNA sequences will be
5   expressed in hosts after the sequences have been operably
linked to an expression control sequence (i.e., positioned to
ensure the translation of the structural gene). These
expression vectors are typically replicable in the host
organisms either as episomes or as an integral part of the host
10   chromosomal DNA. Commonly, expression vectors will contain
selection markers, e.g., tetracycline or neomycin, to permit
detection of those cells transformed with the desired DNA
sequences (see, e.g., U.S. Patent 4,704,362, which is
incorporated herein by reference).

15   In general, prokaryotes can be used for cloning the
DNA sequences encoding the components of bispecific antibodies.
E. coli is one prokaryotic host particularly useful for cloning
the DNA sequences of the present invention. Particular E. coli
strains that can be used include, HB101, DH-1, and MH-1.

20   Other microbial hosts suitable for use include
bacilli, such as Bacillus subtilus, and other
enterobacteriaceae, such as Salmonella, Serratia, and various
Pseudomonas species. In these prokaryotic hosts, one can also
make expression vectors, which will typically contain
25   expression control sequences compatible with the host cell
(e.g., an origin of replication). In addition, any number of a
variety of well-known promoters will be present, such as the
lactose promoter system, a tryptophan (trp) promoter system, a
beta-lactamase promoter system, or a promoter system from phage
30   lambda. The promoters will typically control expression,
optionally with an operator sequence, and have ribosome binding
site sequences and the like, for initiating and completing
transcription and translation.

Other microbes, such as yeast may also be used for
35   expression. Saccharomyces is a preferred host, with suitable
vectors having expression control sequences, an origin of
replication, termination sequences and the like as desired.
Typical promoters include 3-phosphoglycerate kinase and other

15

glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase 2, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

5   When constructing vectors for use in yeast, the plasmid YRp7 can be used (see, Stinchcomb, et al., Nature, 282: 39 (1979)). This plasmid contains the trp1 gene which is a selectable marker for a mutant strain which lacks the ability to grow on media containing tryptophan. The presence of the

10   trp1 gene allows transformed mutant cells to grow on selective media and to be identified.

In addition to microorganisms, mammalian tissue cell culture may also be used to produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH

15   Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma

20   cell lines, etc, but preferably transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen, C. et al., Immunol. Rev. 89:49-68 (1986), which is incorporated herein by reference), and

25   necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, cytomegalovirus, SV40, Adenovirus, Bovine Papilloma

30   Virus, and the like.

Eukaryotic DNA transcription can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting sequences of between 10 to 300bp that increase transcription by a promoter. Enhancers can effectively

35   increase transcription when either 5' or 3' to the transcription unit. They are also effective if located within an intron or within the coding sequence itself. Typically, viral enhancers are used, including SV40 enhancers,

cytomegalovirus enhancers, polyoma enhancers, and adenovirus enhancers. Enhancer sequences from mammalian systems are also commonly used, such as the mouse immunoglobulin heavy chain enhancer.

5   Mammalian expression vector systems will also typically include a selectable marker gene. Examples of suitable markers include, the dihydrofolate reductase gene (DHFR), the thymidine kinase gene (TK), or prokaryotic genes conferring drug resistance. The first two marker genes prefer

10   the use of mutant cell lines that lack the ability to grow without the addition of thymidine to the growth medium. Transformed cells can then be identified by their ability to grow on non-supplemented media. Examples of prokaryotic drug resistance genes useful as markers include genes conferring

15   resistance to G418, mycophenolic acid and hygromycin.
     The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic

20   cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. Other methods used to transform mammalian cells include the use of Polybrene, protoplast fusion, liposomes and microinjection (see, generally, Sambrook et al., supra).

25   Once expressed, bispecific antibodies, epitope binding components, their dimers, or individual light and heavy chains with or without linked leucine zippers, or individual leucine zipper species themselves can be purified according to standard procedures of the art, including ammonium sulfate

30   precipitation, fraction column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically or in developing

35   and performing assay procedures, immunofluorescent stainings, and the like (see, generally, Immunological Methods, Vols. I and II, Eds. Lefkovits and Pernis, Academic Press, New York, N.Y. (1979 and 1981)).

The bispecific antibodies of the present invention can be used for therapy. By way of illustration and not limitation, they can be used to treat cancer, autoimmune diseases, or viral infections. For treatment of cancer, one of

5 the epitope binding components will typically bind to an antigen expressed preferentially on cancer cells, such as erbB-2, CEA, CD33, and many other antigens well known to those skilled in the art. For treatment of autoimmune disease, one of the epitope binding components will typically bind to an

10 antigen expressed on T-cells, such as CD4, the IL-2 receptor, the various T-cell antigen receptors and many other antigens well known to those skilled in the art (e.g., see Fundamental Immunology, 2nd ed., W.E. Paul, ed., Raven Press: New York, NY, which is incorporated herein by reference). For treatment of

15 viral infections, one of the epitope binding components will typically bind to an antigen expressed on cells infected by a particular virus such as the various glycoproteins (e.g., gB, gD, gH) of herpes simplex virus and cytomegalovirus, and many other antigens well known to those skilled in the art (e.g.,

20 see Virology, 2nd ed., B.N. Fields et al., eds., (1990), Raven Press: New York, NY, which is incorporated herein by reference). In any case, the second epitope binding component will typically bind to an epitope expressed on T cells or other white blood cells that is capable of transmitting an

25 activating signal, such as CD3 or CD16.

Pharmaceutical compositions comprising bispecific antibodies of the present invention are useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration

30 will commonly comprise a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of

35 particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions

such as pH adjusting and buffering agents, toxicity adjusting
agents and the like, for example sodium acetate, sodium
chloride, potassium chloride, calcium chloride, sodium lactate,
etc. The concentration of the bispecific antibodies in these

5   formulations can vary widely, i.e., from less than about 0.01%,
usually at least about 0.1% to as much as 5% by weight and will
be selected primarily based on fluid volumes, viscosities,
etc., in accordance with the particular mode of administration
selected.

10   Thus, a typical pharmaceutical composition for
intramuscular injection could be made up to contain 1 ml
sterile buffered water, and about 1 mg of bispecific antibody.
A typical composition for intravenous infusion can be made up
to contain 250 ml of sterile Ringer's solution, and 10 mg of

15 bispecific antibody. Actual methods for preparing parenterally
administrable compositions will be known or apparent to those
skilled in the art and are described in more detail in, for
example, Remington's Pharmaceutical Science, 15th Ed., Mack
Publishing Company, Easton, Pennsylvania (1980), which is

20 incorporated herein by reference.
        The bispecific antibodies of this invention can be
lyophilized for storage and reconstituted in a suitable carrier
prior to use. This technique has been shown to be effective
with conventional immune globulins and art-known lyophilization

25 and reconstitution techniques can be employed. It will be
appreciated by those skilled in the art that lyophilization and
reconstitution can lead to varying degrees of antibody activity
loss (e.g., with conventional immune globulins, IgM antibodies
tend to have greater activity loss than IgG antibodies) and

30 that use levels may have to be adjusted to compensate.
        The compositions containing the present bispecific
antibodies or a cocktail thereof can be administered for
prophylactic and/or therapeutic treatments. In therapeutic ap-
plication, compositions are administered to a patient already

35 affected by the particular disease, in an amount sufficient to
cure or at least partially arrest the condition and its
complications. An amount adequate to accomplish this is
defined as a "therapeutically effective dose." Amounts

effective for this use will depend upon the severity of the
condition and the general state of the patient's own immune
system, but generally range from about 0.01 to about 100 mg of
bispecific antibody per dose, with dosages of from 1 to 10 mg
5   per patient being more commonly used.

In prophylactic applications, compositions containing
the bispecific antibodies or a cocktail thereof are
administered to a patient not already in a disease state to
enhance the patient's resistance. Such an amount is defined to
10 be a "prophylactically effective dose." In this use, the
precise amounts again depend upon the patient's state of health
and general level of immunity, but generally range from 0.1 to
100 mg per dose, especially 1 to 10 mg per patient.

Single or multiple administrations of the
15 compositions can be carried out with dose levels and pattern
being selected by the treating physician. In any event, the
pharmaceutical formulations should provide a quantity of the
bispecific antibodies of this invention sufficient to
effectively treat the patient.

20 The bispecific antibodies described herein can also
be used to cross-link an epitope binding component and a
detection agent such as ferritin (Hammerling et al., (1968) J.
Exp. Med. 128: 1461) or horseradish peroxidase (Milstein and
Cuello, (1983), Nature 305: 537) for diagnosis and imaging
25 purposes. The detection agent can be linked to an epitope
binding component through the second epitope binding component
of the bispecific antibody, or be linked directly to the first
epitope binding component by the heterodimer forming leucine
zippers described herein. Similarly, metallothionein, a
30 protein that binds heavy metal atoms, can be expresed with Fos
leucine zipper as fusion protein and linked to Fab'-Jun. The
resulting product can be used to deliver radionuclides to the
antigen bearing site for imaging and therapy. Similarly, by
way of example and not limitation, a protein toxin such as
35 ricin or Pseudomonas aeruginosa exotoxin can also be fused with
a Fos leucine zipper and then linked to a Fab'-Jun to be used
as an immunotoxin.

Kits can also be supplied for use with the subject
bispecific antibodies in the protection against or detection of
a cellular activity or for the presence of a selected cell
surface receptor. Thus, the subject composition of the present
5 invention may be provided, usually in a lyophilized form in a
container, either alone or in conjunction with additional
antibodies specific for the desired cell type. The bispecific
antibodies, which may be conjugated to a label or toxin, or
unconjugated, are included in the kits with buffers, such as
10 Tris, phosphate, carbonate, etc., stabilizers, biocides, inert
proteins, e.g., serum albumin, or the like, and a set of
instructions for use. Generally, these materials will be
present in less than about 5% wt. based on the amount of active
antibody, and usually present in total amount of at least about
15 0.001% wt. based again on the antibody concentration.
Frequently, it will be desirable to include an inert extender
or excipient to dilute the active ingredients, where the
excipient may be present in from about 1 to 99% wt. of the
total composition. Where a second antibody capable of binding
20 to the bispecific antibody is employed in an assay, this will
usually be present in a separate vial. The second antibody is
typically conjugated to a label and formulated in an analogous
manner with the antibody formulations described above.

The following examples are offered by way of
25 illustration, not by way of limitation.

EXPERIMENTAL

Construction of plasmids. Genes for the leucine
zipper portions of Fos and Jun were separately synthesized,
30 using four overlapping synthetic oligonucleotides (model 380B
DNA synthesizer, Applied Biosystems, Foster City, CA). Each of
the genes was then fused in phase to the first codon of the C_H2
exon of the mouse IgG2a gene (Fig. 1) using the polymerase
chain reaction (PCR) method (Saiki et al., (1988) Science
35 239:487) described by Yon and Fried (Yon and Fried, (1989)
Nucl. Acid Res. 17:4849). The resulting PCR products were 911
bp XhoI-SaII fragments, encompassing part of the C_H1 exon, the
C_H1:H intron, the hinge (H) exon, the H:C_H2 intron, and the

C_H2/zipper exon. The XhoI site is the natural restriction site
within the C_H1 exon, but the SalI site was added to the end of
the zipper sequences during PCR. A 162 bp SalI-BamHI fragment
containing the 3' noncoding sequence of the mouse IgG2a gene

5   was also generated by PCR. This sequence begins immediately 3'
to the stop codon of the C_H3 exon and provides the
polyadenylation signal. For the Jun and Fos constructs, the
XhoI-SalI and the SalI-BamHI fragments were then inserted
together between the XhoI and BamHI sites of a mouse heavy

10   chain expression vector, replacing the C_H2 and the C_H3 exons
with the C_H2/zipper exon (Fig. 2). The heavy chain expression
vector, which contains the mutant dihydrofolate reductase gene
(mdhfr) as the selectable marker (Simonsen and Levinson, (1983)
Proc. Natl. Acad. Sci. USA 80:2495), the human cytomegalovirus

15   (hCMV) major immediate early promoter and enhancer for
transcription initiation (Boshart et al., (1985) Cell 41:521),
and the mouse IgG2a constant region, was constructed from the
respective fragments by standard methods.

     A XbaI fragment containing the V_H exon of the mouse
20   anti-Tac heavy chain gene (Queen et al., (1989) Proc. Natl.
Acad. Sci. USA 86:10029) was then inserted into the XbaI site
of the Jun zipper containing vector (Fig. 2) to produce plasmid
pTAC-Jun. Similarly, the V_H gene of the hamster antibody 145-
2C11 heavy chain gene was inserted in the Fos zipper containing

25   vector to produce plasmid p145-2C11-Fos. For light chain
expression, two vectors were used that contain the hCMV
promoter and enhancer and the murine C_L gene including part of
the preceding intron. One of these vectors incorporates the
xanthine-guanine phosphoribosyl transferase (gpt) gene

30   (Mulligan and Berg, (1981) Proc. Natl. Acad. Sci. USA 78:2072)
and the other the hygromycin B phosphotransferase (hyg) gene
(Blochlinger and Diggelmann, (1984) Mol. Cell. Biol. 4:2929).
The vectors were constructed from the respective fragments by
standard methods. XbaI sites were added to the V_L gene

35   fragments of anti-Tac and 145-2C11 by PCR. The V_L gene of
anti-Tac was cloned in the gpt containing vector and the V_L
gene of 145-2C11 in the hyg containing vector to generate the
respective plasmids pTAC-k and p145.2C11-k.

<u>Transfection</u>. Transfection was by electroporation
using a Gene Pulser apparatus (Bio-Rad, Richmond, CA) at 360 V
and 25 μFD capacitance according to the manufacturer's
instructions. Before transfection, the light chain and heavy
5 chain containing plasmids were linearized using Fsp1, extracted
with phenol-chloroform, and ethanol precipitated. All
transfections were done using 20 μg of each plasmid DNA and
about 10^7 Sp2/0 cells (ATCC CRL 1581) in phosphate-buffered
saline (PBS). The cells from each transfection were plated
10 into one 96-well tissue culture plate. After 48 hr, selective
medium was applied: DMEM + 10% fetal calf serum (FCS) + either
HT media supplement (Sigma, St. Louis, MO) plus 300 μg/ml
xanthine with 1 μg/ml mycophenolic acid or 500 μg/ml hygromycin
(Boehringer Mannheim Biochemicals, Indianapolis, IN). After
15 the wells had become confluent with surviving colonies of
cells, medium from each well was assayed for the presence and
quantity of secreted antibodies by ELISA using goat anti-mouse
gamma Ig (Sigma).

<u>Flow Cytometry</u>. 2.5 x 10^4 Hut-102 cells were
20 incubated with various concentrations of anti-Tac, anti-Tac-
Jun, or bispecific F(ab'-zipper)$_2$ in 100 μl of PBS at 4°C for
30 min. Cells were then washed in PBS, resuspended in 25 μl of
PBS containing 50 ng of FITC-conjugated rat anti-mouse kappa
(Pandex, Mundelein, IL), and incubated at 4°C for 30 min.
25 Cells were washed with PBS, fixed in 1% paraformaldehyde and
analyzed by FACScan (Becton Dickinson, Mountain View, CA). The
binding of anti-CD3 or its derivatives to EL4 cells was
similarly analyzed. To estimate the concentration of
bispecific F(ab'-zipper)$_2$, a standard curve of fluorescence
30 intensity vs anti-CD3 antibody concentration was used.

<u>F(ab'-zipper)$_2$ purification</u>. Media supernatant from
transfectants was passed over a column of monoclonal rat anti-
mouse kappa Sepharose (Zymed, South San Francisco, CA), and
bound proteins were eluted with 0.2 M glycine-HCl, pH 2.1. The
35 eluted fractions were neutralized with Tris base and dialyzed
against PBS. In one experiment, concentrated medium from a
supertransfectant was adjusted to pH 5.2 by 1:4 dilution in 10
mM MES buffer and was loaded onto a BAKERBOND ABx column (J. T.

Baker, Phillsburg, NJ) for separation on a FPLC system
(Pharmacia LKB Biotechnology, Piscataway, NJ). Bound proteins
were eluted with a linear gradient of 0-0.25 M $(NH_4)_2SO_4$, and
F(ab'-zipper)$_2$ proteins were identified by ELISA or flow

5 cytometry as described above. Bispecific F(ab'-zipper)$_2$ formed
in vitro was similarly purified by ABx chromatography. F(ab'-
zipper)$_2$ concentration in impure fractions was estimated by
flow cytometry as described above. In pure protein fractions
the concentration was determined by absorbance at 280 nm,

10 assuming that 1 mg/ml has an A_{280} of 1.4.

Formation of bispecific F(ab'-zipper) in vitro.
Homodimers of anti-Tac-Jun and anti-CD3-Fos were reduced with
2-mercaptoethanylamine in PBS at 37°C for 1 hr to form Fab'-
zippers. They were then mixed and dialyzed against redox

15 buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 500 µM reduced
glutathione and 500 µM oxidized glutathione) for 48 hr at 4°C,
and the buffer was changed back to PBS by dialysis.

Cytotoxicity assay with mouse effector cells. Mouse
spleen cells were cultured in DMEM + 10% FCS + 4 µg/ml

20 Concanavalin A. After 3 days, the cells were passaged 1:2 in
DMEM + 10% FCS + 10 U/ml recombinant IL-2 (Amgen, Thousand
Oaks, CA). The effector cells were harvested 4 days later and
used in the cytotoxicity assay. Target cells were prepared by
incubating HuT-102 cells with 100 µCi Na$_2$^{51}CrO$_4$ (Amersham,

25 Chicago, IL) in 100 µl of DMEM at 37°C for 1 h. The cells were
washed 2 times with DMEM before use. Cytotoxicity was measured
by a standard ^{51}Cr-release assay in a 96-well tissue culture
plate. Each well received 50 µl of bispecific F(ab'-zipper)$_2$
in PBS, 10^4 ^{51}Cr-labeled HuT-102 cells in 50 µl of DMEM, and 50

30 µl of effector cells in DMEM. Total volume in each well was
200 µl. The cell mixtures were incubated at 37°C for 4 h to
allows lysis. After centrifugation, supernatant was removed
from each well to assay for the release of ^{51}Cr from HuT-102
cells. Percentages of specific release in the cytotoxicity

35 assay were calculated as: {Counts released by bispecific
F(ab'-zipper)$_2$ minus counts released without added F(ab'-
zipper)$_2$} / {Counts released by 0.1% SDS minus counts released
without added F(ab'-zipper)$_2$} x 100. All points in the

cytotoxicity assay were determined in triplicate and their
means were plotted.

      <u>Construction and transfection of Fab'-leucine zipper</u>

<u>genes</u>. We used the PCR method to join the Jun or Fos leucine

5 zipper sequence to the first codon of the C_H2 exon of a mouse
IgG2a gene (Fig. 1). At the fusion junctions, two glycine
codons were introduced to make the joints more flexible in the
protein products. After the leucine zipper sequences, a stop
codon and a sequence containing the polyadenylation signal from

10 the mouse IgG2a gene were included. The gene fusions were
separately inserted into an expression vector previously used
for heavy chain synthesis (Fig. 2). V_H genes for desired
antibodies can be inserted in the XbaI sites of the resulting
new vectors. It is expected that mRNA transcripts will then

15 initiate from the CMV promoter on each plasmid, and the V_H,
C_H1, hinge and C_H2/leucine zipper exons will be spliced
together.

      In the Jun expression plasmid we inserted the V_H gene
of the mouse anti-Tac antibody, and in the Fos plasmid, the V_H

20 gene of the hamster 145-2C11 antibody. The anti-Tac antibody
binds to the p55 chain of the human Il-2 receptor (IL-2R)
(Uchiyama et al., (1981) <u>J. Immunol.</u> <u>126</u>:1393); its heavy and
light chain genes were previously cloned (Queen et al., (1989)
<u>Proc. Natl. Acad. Sci. USA</u> <u>86</u>:10029). The 145-2C11 antibody

25 recognizes the ϵ chain of the mouse CD3 complex (Leo et al.,
(1987) <u>Proc. Natl. Acad. Sci. USA</u> <u>84</u>:1374); its heavy and light
chain genes were also cloned. Each V_H gene included the signal
sequences and J segment, and was followed by a splice donor
sequence to allow splicing to the C_H1 domain (Queen et al.,

30 (1989) <u>Proc. Natl. Acad. Sci. USA</u> <u>86</u>:10029). Analogous
plasmids were prepared that respectively contained the V_L genes
of anti-Tac and 145-2C11 together with the mouse V_κ gene (Fig.
2).

      Each heavy chain expression plasmid was cotransfected

35 with the corresponding light chain plasmid into the murine
myeloma cell line Sp2/0. Stable transfectants were selected
using the gpt marker for anti-Tac-Jun, and the hyg marker for
anti-CD3-Fos. Media supernatant from each transfectant was

screened for the presence of antibody protein by ELISA with
goat anti-mouse gamma antibody. ELISA-positive transfectants
were confirmed by using flow cytometry to test their
supernatants for the presence of antibody-like molecules
5   binding to CD3 and p55 positive cell lines. In both cases,
transfectants were obtained at a frequency of about 1 per 10^5
myeloma cells. The transfectants secreted about 0.1-2
$\mu g/ml/10^6$ cells/24 h of F(ab')2-like molecule. The level of
production of these molecules appears to be slightly lower than
10   that of whole antibody molecules in similar experiments. The
higher yielding transfectants for anti-Tac-Jun and anti-CD3-Fos
were expanded for further characterization.

      Purification and characterization of anti-Tac-Jun and
anti-CD3-Fos. Affinity chromatography was used to purify anti-
15   Tac-Jun and anti-CD3-Fos. Supernatant from each kind of
transfectant was loaded onto a rat anti-mouse kappa Sepharose
column. Proteins that bound to the column were eluted with
glycine-HCl at pH 2.1. After dialysis against PBS, the eluted
proteins were analyzed on an SDS PAGE gel (Laemmli, (1970)
20   Nature 227:680) with or without reduction (Fig. 3). The
reduced proteins displayed only 2 bands, of apparent molecular
weights 25 kd and 31 kd, corresponding respectively to the
light chain and the heavy chain Fd-zipper. The nonreduced
proteins displayed major bands of approximate molecular weights
25   25 kd or less and 100 kd or more. In light of the fact that
both bands can be reduced to light chain and Fd-zipper, they
most likely represent free light chain (25 kd), and F(ab'-
zipper)$_2$ dimers of anti-Tac-Jun and anti-CD3-Fos. In addition,
the anti-CD3-Fos sample contained a 50 kd band, which consisted
30   of light chain dimers, as verified below. It should be
mentioned that the affinity chromatography used to purify the
proteins would capture free light chains and light chain dimers
but not free Fd-zipper chains. So it is highly unlikely that
this 50 kd protein is the Fd-zipper dimer. The influence of
35   intrachain disulfide bonds on the mobility of the proteins on
SDS PAGE can be observed by comparing the degree of light chain
migration with or without reduction.

Formation of bispecific F(ab'-zipper)₂ _in vivo_.

Having shown that the plasmid constructs could direct expression of appropriate $F(ab'-zipper)_2$ homodimers, we then showed that bispecific $F(ab'-zipper)_2$ could be produced _in vivo_

5   by expressing all four different polypeptide chains needed for anti-Tac-Jun and anti-CD3-Fos in one transfectant. An anti-CD3-Fos transfectant was further transfected with the plasmid constructs for anti-Tac-Jun. Supertransfectants were selected using both the _gpt_ and the _hyg_ markers and screened by ELISA

10 for secretion of antibody proteins that could bind to purified IL-2R p55. Supernatants from these supertransfectants were also analyzed by flow cytometry for the presence of anti-Tac as well as anti-CD3 activities.

A representative supertransfectant producing both

15 anti-Tac and anti-CD3 activity was expanded to further characterize its antibody products. Media supernatant from this supertransfectant was analyzed by FPLC chromatography on an ABx column, eluting with a $(NH_4)_2SO_4$ gradient (Fig. 4A). Only five peaks contained proteins reactive in an ELISA assay

20 for mouse heavy and light chain were eluted (Fig. 4B). The antibody-positive peaks, designated as fractions I, II, III, IV, and V, were assayed by flow cytometry for binding to CD3⁺ EL4 T-lymphoma cells and IL-2R⁺ HuT-102 cells. Fraction II contained both anti-Tac and anti-CD3 activities, whereas

25 fraction I contained mostly anti-Tac activity and fractions III and IV and V anti-CD3 activity only (Fig. 4C). In other experiments, media from transfectants separately expressing anti-Tac-Jun and anti-CD3-Fos was chromatographed in the same system: anti-Tac-Jun eluted in the same position as fraction I

30 and anti-CD3-Fos in the position of fraction IV (data not shown). So fraction II contains bispecific antibody and fraction III and V other hybrid antibodies having only anti-CD3 activity.

To demonstrate that fraction II indeed contained

35 bispecific activity, we used it to mediate the lysis of ⁵¹Cr-labeled HuT-102 cells by activated mouse splenic T cells (Fig. 5). Protein in fraction II would lyse the target cells down to a concentration below 20 ng/ml. Purified anti-CD3-Fos and

27

anti-Tac-Jun homodimers, either alone or in combination, were totally ineffective in lysing the cells. These data indicate that bispecific activity could indeed be formed *in vivo*.

5   Formation of bispecific F(ab'-zipper)$_2$ *in vitro*. The homodimeric F(ab'-zipper)$_2$ proteins could be reduced at the hinge region to form Fab'-zipper monomers. Various concentrations of 2-mercaptoethylamine were used to determine the best conditions to form Fab'-zipper without the

10   dissociation of light chain from the heavy chain Fd-zipper. The reduction products were analyzed on SDS PAGE under nonreducing conditions. The best conditions for the reduction of the purified anti-Tac-Jun was with 4 mM 2-mercaptoethylamine in PBS at 37°C for 1 h. Anti-CD3-Fos required 2 mM 2-

15   mercaptoethylamine under the same conditions. The proteins reduced under these conditions are shown in Fig. 6. In both cases a set of protein bands of 50-55 kd, corresponding to the Fab'-zipper, appeared upon reduction. The reason for the heterogeneity is unclear, but it has been observed before by

20   others (Curran and Franza, (1988) <u>Cell</u> <u>55</u>:395). The light chain dimer in the anti-CD3-Fos sample was also reduced into monomer very readily. Under these conditions there was minimum dissociation of the light chain from the Fab'-zipper, as evidenced by the absence of the Fd-zipper band (compare with

25   Fig. 3).
          The anti-Tac and anti-CD3 Fab'-zipper proteins were mixed together at a 1:1 ratio and a final concentration of 100 μg/ml, dialyzed against a redox buffer, and then dialyzed against PBS. The Fab'-zipper bands disappeared, and a new band

30   corresponding to a F(ab'-zipper)$_2$ protein appeared (Fig. 6, lane 5). The new proteins were fractionated by ABx chromatography under the same conditions used for the F(ab'-zipper)$_2$ produced *in vivo*. There were three major and two minor protein peaks in the chromatogram (Fig. 7A). A

35   combination of ELISA, flow cytometry (Fig. 7B), and SDS PAGE (Fig. 8) was used to identify the peaks. The flow through (FT) fraction contained excess light chains and light chain dimers only. The first eluted major peak (fraction I) contained

mostly light chains but also a minor amount of anti-Tac-Jun detected by flow cytometry. In addition, this fraction, as well as the following fractions, contained BSA, which was used to pretreat the ABX column to prevent nonspecific protein

5 sticking. The second eluted peak (fraction II) had minor amounts of both anti-Tac and anti-CD3 activities according to flow cytometry, and was composed of F(ab'-zipper)$_2$, partly in aggregated forms. The last peak (fraction IV) was identified as anti-CD3-Fos because of its elution position (see fraction

10 IV, Fig. 4A) and its EL4 cell binding activity.

The third major peak (fraction III) on the chromatogram eluted precisely in the same position as the *in vivo* produced bispecific F(ab'-zipper)$_2$ (Fig. 4A, fraction II). This fraction binds to both CD3$^+$ EL4 cells and IL-2R$^+$ HuT-102

15 cells. Only one principle protein band was observed on SDS PAGE of this fraction (Fig. 8A, lane 4), with the molecular weight expected for F(ab'-zipper)$_2$. Upon reduction, it dissociated into two closely migrating Fd-zipper bands and two distinct light chain bands in roughly equal amounts (Fig. 8B,

20 lane 4). By running anti-CD3-Fos homodimers side by side with this sample, we were able to identify the upper Fd-zipper band as anti-CD3-Fos Fd-zipper and the upper light chain band as anti-CD3-Fos light chain (compare lane 4 and 5 in Fig. 8B). Because anti-Tac-Jun and anti-CD3-Fos homodimers were shown to

25 elute in other fractions, fraction III must consist of heterodimers of anti-Tac-Jun with anti-CD3-Fos. To confirm that this fraction contains bispecific antibody, we used it for targeted killing of Hut-102 cells by mouse T cells. Specific lysis was seen at a concentration of less than 10 ng/ml (Fig.

30 9). Taken together, these data show that the F(ab'-zipper)$_2$ formed *in vitro* by disulfide exchange is mainly the bispecific anti-Tac x anti-CD3, with little side product formation.

From the foregoing, it will be appreciated that the bispecific antibodies of the present invention offer numerous

35 advantages over other bispecific antibodies. In comparison to bispecific antibodies that are not formed by pairwise linkage of two leucine zippers, the present bispecific antibodies can be more economically produced in highly purity and yield. This

improved purity and yield will permit bispecific antibodies
that can be used for therapy of human or veterinary disease, or
for prevention of disease.

     Although the present invention has been described in

5 some detail by way of illustration and example for purposes of
clarity and understanding, it will be apparent that certain
changes and modifications may be practiced within the scope of
the appended claims.

10

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Tso, J. Y.
                Kostelny, Sheri A.
                Cole, Michael S.

    (ii) TITLE OF INVENTION: Bispecific Antibody Heterodimers

   (iii) NUMBER OF SEQUENCES: 5

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Tracy J. Dunn
        (B) STREET: One Market Plaza, Steuart Tower, Suite 2000
        (C) CITY: San Francisco
        (D) STATE: California
        (E) COUNTRY: USA
        (F) ZIP: 94105

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: US 07/801,798
        (B) FILING DATE: 29-NOV-1991
        (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Dunn, Tracy J.
        (B) REGISTRATION NUMBER: 34,587
        (C) REFERENCE/DOCKET NUMBER: 11823-32

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 415-326-2400
        (B) TELEFAX: 415-326-2422

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Region
        (B) LOCATION: 2..7
        (D) OTHER INFORMATION: /note= "Residues 2-7 can be any of
            the 20 naturally ocurring amino acids."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Xaa Xaa Xaa Xaa Xaa Xaa
1   5

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 151 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
      (A) NAME/KEY: intron
      (B) LOCATION: 1..16

    (ix) FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 17..143

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
CCATCTCTCC TCATCA GCA GGC GGC CGC ATC GCC CGG CTC GAG GAA AAA
    49
                   Ala Gly Gly Arg Ile Ala Arg Leu Glu Glu Lys
                    1               5                      10

GTG AAA ACC TTG AAA GCT CAG AAC TCG GAG CTC GCG TCC ACG GCC AAC
    97
Val Lys Thr Leu Lys Ala Gln Asn Ser Glu Leu Ala Ser Thr Ala Asn
             15              20              25

ATG CTC AGG GAA CAG GTG GCA CAG CTT AAA CAG AAA GTC ATG AAC T
    143
Met Leu Arg Glu Gln Val Ala Gln Leu Lys Gln Lys Val Met Asn
         30              35              40

GAGTCGAC
    151
```

33

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 42 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Ala Gly Gly Arg Ile Ala Arg Leu Glu Glu Lys Val Lys Thr Leu Lys
 1               5               10                  15

Ala Gln Asn Ser Glu Leu Ala Ser Thr Ala Asn Met Leu Arg Glu Gln
                20              25                  30

Val Ala Gln Leu Lys Gln Lys Val Met Asn
        35                  40
```

| (2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 151 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (ix) FEATURE:
          (A) NAME/KEY: intron
          (B) LOCATION: 1..16

     (ix) FEATURE:
          (A) NAME/KEY: CDS
          (B) LOCATION: 17..143

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCATCTCTCC TCATCA GCA GGC GGG TTA ACT GAT ACA CTC CAA GCG GAG
     49
                   Ala Gly Gly Leu Thr Asp Thr Leu Gln Ala Glu
                    1   5   10

ACC GAC CAG CTG GAA GAT AAG AAG TCT GCT CTG CAG ACC GAG ATT GCC
     97
Thr Asp Gln Leu Glu Asp Lys Lys Ser Ala Leu Gln Thr Glu Ile Ala
             15   20   25

AAC CTG CTG AAG GAG AAG GAA AAA CTG GAG TTC ATC CTG GCC GCC T
    143
Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala
             30   35   40

GAGTCGAC
    151

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ala Gly Gly Leu Thr Asp Thr Leu Gln Ala Glu Thr Asp Gln Leu Glu
 1               5                  10                  15

Asp Lys Lys Ser Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu
               20                  25                  30

Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala
           35                  40
```

36

WHAT IS CLAIMED IS:

      1.   A bispecific antibody, comprising:

      a first protein comprising a first leucine zipper
linked to a first epitope binding component; and

      a second protein comprising a second leucine zipper
linked to a second epitope binding component, wherein said
second leucine zipper has pairwise affinity for said first
leucine zipper.

      2.   A bispecific antibody of claim 1, wherein one
leucine zipper is a Fos leucine zipper.

      3.   A bispecific antibody of claim 1, wherein one
leucine zipper is a Jun leucine zipper.

      4.   A bispecific antibody, comprising:

      a first protein comprising a Fos leucine zipper
linked to a first epitope binding component; and

      a second protein comprising a Jun leucine zipper
linked to a second epitope binding component.

      5.   A bispecific antibody of claim 1, wherein said
first and second leucine zippers both are of the structural
formula (leucine-X_1-X_2-X_3-X_4-X_5-X_6)$_n$, wherein each of X_1, X_2, X_3,
X_4, X_5, and X_6 is selected from the group consisting of the
conventional 20 amino acids and n is an integer that is at
least 3.

      6.   A bispecific antibody of claim 1, wherein said
first leucine zipper has the amino acid sequence shown in Fig.
1(b) and said second leucine zipper has the amino acid sequence
shown in Fig. 1(a).

      7.   A bispecific antibody of claim 1, wherein the
first epitope binding component binds to a human IL-2 receptor
protein with an affinity of about 10^8 M^{-1} or greater.

8.   A bispecific antibody of claim 1, wherein the second epitope binding component binds to human CD3 protein with an affinity of about 10^8 M^{-1} or greater.

9.   A bispecific antibody of claim 1, wherein the first epitope binding component is an Fab'.

10.   A bispecific antibody of claim 1, wherein at least one said epitope binding component is a humanized immunoglobulin.

11.   A method for preparing bispecific antibodies, said method comprising:
        producing a first protein comprising a first leucine zipper linked to a first epitope binding component capable of binding a first epitope;
        producing a second protein comprising a second leucine zipper joined to a second epitope binding component capable of binding a second epitope, wherein said second leucine zipper has pairwise affinity for said first leucine zipper; and
        contacting said first protein with said second protein in conditions that permit formation of a F(ab'-zipper)$_2$ heterodimer to form said bispecific antibody.

12.   A method according to claim 11, wherein one leucine zipper is a Fos or Jun leucine zipper.

13.   A method according to claim 11, wherein one leucine zipper is a Fos leucine zipper and the other leucine zipper is a Jun leucine zipper.

14.   A method according to claim 11, wherein said first leucine zipper and said second leucine zipper both comprise an amino acid sequence corresponding to a structural formula (leucine-X_1-X_2-X_3-X_4-X_5-X_6)$_n$, wherein each of X_1, X_2, X_3, X_4, X_5, and X_6 is selected from the group consisting of the

conventional 20 amino acids and n is an integer that is at least 3.

15. A method according to claim 11, wherein said
contacting of the first protein with the second protein occurs
in vitro.

16. A method according to claim 11, wherein said
contacting of the first protein with the second protein occurs
in vivo in a single cell that expresses the first and the
second protein.

17. A method for for preparing bispecific
antibodies, said method comprising:
    producing a first protein comprising a first leucine
zipper linked to a first epitope binding component capable of
binding a first epitope;
    producing a second protein comprising a second
leucine zipper joined to a second epitope binding component
capable of binding a second epitope, wherein said second
leucine zipper has pairwise affinity for said first leucine
zipper;
    contacting said first protein with said second
protein to form a heterodimer;
    forming a direct linkage between the first epitope
binding component and the second epitope binding component,
wherein after said direct linkage is formed, leucine zipper
sequences can be separated from epitope binding components and
said direct linkage is retained;
    cleaving a linkage between said first leucine zipper
and said first epitope binding component; and
    cleaving a linkage between said second leucine zipper
and said second epitope binding component to form a bispecific
antibody comprising a heterodimer of a first epitope binding
component linked to a second epitope binding component.

18.   A method according to claim 17, wherein at least one epitope binding component is an Fab'.

19.   A method according to claim 17, wherein said linkage between said first epitope binding component and said second epitope binding component is a disulfide bond.

20.   A method according to claim 17, further comprising a step of isolating the linked first and second epitope binding components away from said cleaved first and second leucine zippers.

21.   A method according to claim 17, wherein cleavage of a leucine zipper from an epitope binding component occurs at a linkage comprising an asparagine-glycine peptide bond which can be cleaved by hydroxylamine.

22.   A composition comprising a polynucleotide encoding a polypeptide comprising an epitope binding component and a leucine zipper.

23.   A composition of claim 22 wherein the leucine zipper is the Jun or Fos leucine zipper.

24.   A polynucleotide that encodes a leucine zipper and an epitope binding component comprising the V, C_{H1}, and hinge domains of an antibody heavy chain.

25.   A polynucleotide of claim 23, wherein the encoded epitope binding component is a Fab'.

```
  1 CCATCTCTCCTCATCA GCA  GGC  GGC  CGC  ATC  GCC  CGG  CTC  GAG  GAA
                          Ala  Gly  Gly  Arg  Ile  Ala  Arg  Leu  Glu  Glu
       H:C 2 INTRON    1  Ala
           H              CH2                    JUN LEUCINE ZIPPER
```

```
 47 AAA  GTG  AAA  ACC  TTG  AAA  GCT  CAG  AAC  TCG  GAG  CTC  GCG  TCC
 11 Lys  Val  Lys  Thr  Leu  Lys  Ala  Gln  Asn  Ser  Glu  Leu  Ala  Ser
```

```
 89 ACG  GCC  AAC  ATG  CTC  AGG  GAA  CAG  GTG  GCA  CAG  CTT  AAA  CAG
 25 Thr  Ala  Asn  Met  Leu  Arg  Glu  Gln  Val  Ala  Gln  Leu  Lys  Gln
```

```
                               SalI
131 AAA  GTC  ATG  AAC  TGA  GTCGAC
 39 Lys  Val  Met  Asn  STOP
```

FIG. 1a.

```
  1 CCATCTCTCCTCATCA GCA  GGC  GGG  TTA  ACT  GAT  ACA  CTC  CAA  GCG
                          Ala  Gly  Gly  Leu  Thr  Asp  Thr  Leu  Gln  Ala
       H:C 2 INTRON    1  Ala
           H              CH2                    FOS LEUCINE ZIPPER
```

```
 47 GAG  ACC  GAC  CAG  CTG  GAA  GAT  AAG  AAG  TCT  GCT  CTG  CAG  ACC
 11 Glu  Thr  Asp  Gln  Leu  Glu  Asp  Lys  Lys  Ser  Ala  Leu  Gln  Thr
```

```
 89 GAG  ATT  GCC  AAC  CTG  CTG  AAG  GAG  AAG  GAA  AAA  CTG  GAG  TTC
 25 Glu  Ile  Ala  Asn  Leu  Leu  Lys  Glu  Lys  Glu  Lys  Leu  Glu  Phe
```

```
                               SalI
131 ATC  CTG  GCC  GCC  TGA  GTCGAC
 39 Ile  Leu  Ala  Ala  STOP
```
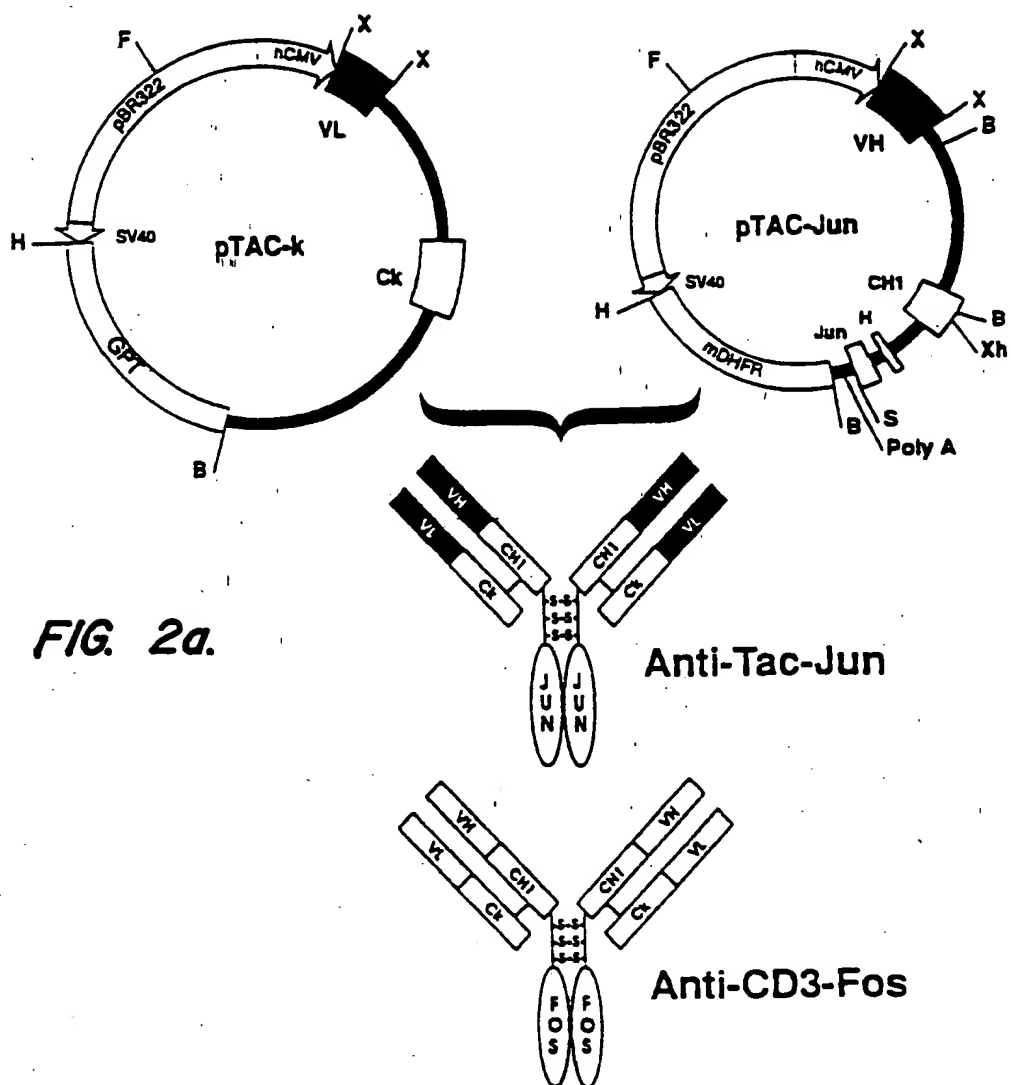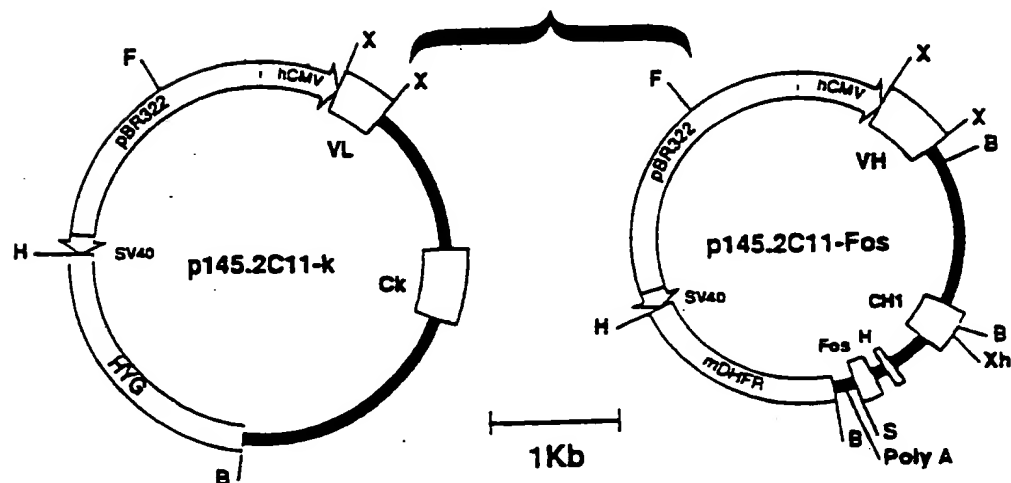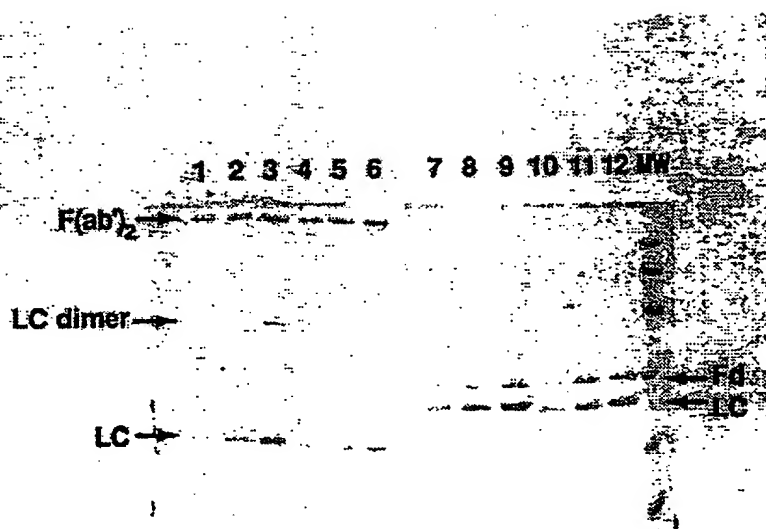
FIG. 1b.

FIG. 2a.

Anti-Tac-Jun

Anti-CD3-Fos

1Kb

FIG. 2b.

FIG. 3.

FIG. 4a.

FIG. 4b.

FIG. 4c.

ABX Column Fraction Number

FIGURE 5

6/10

FIG. 6.

FIG. 7a.

FIG. 7b.

ABX Column Fraction Number
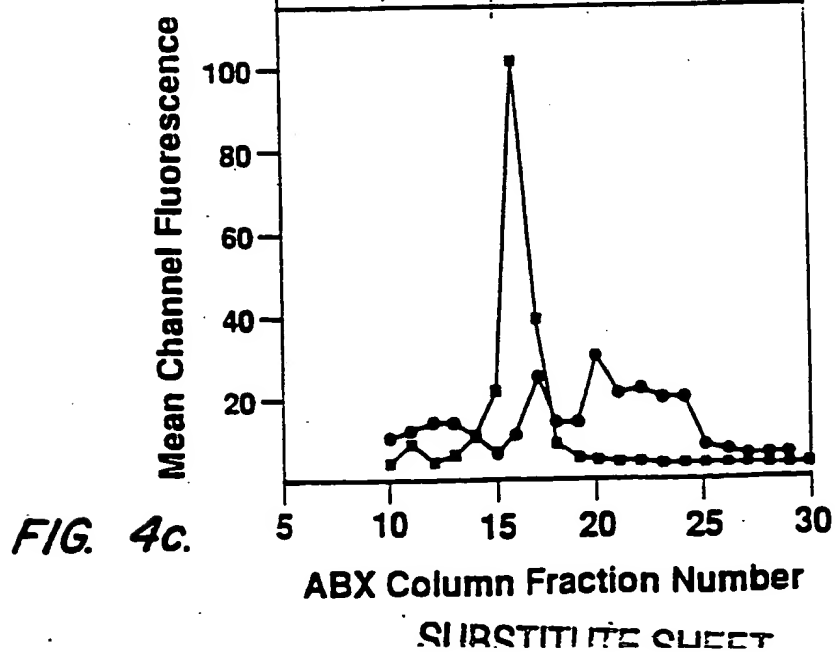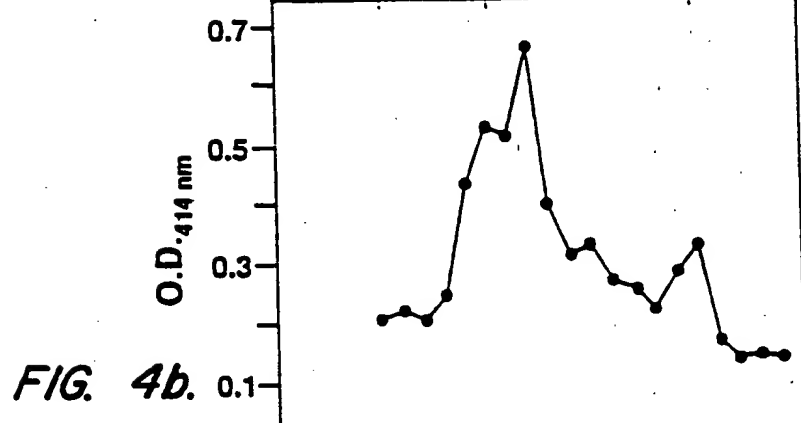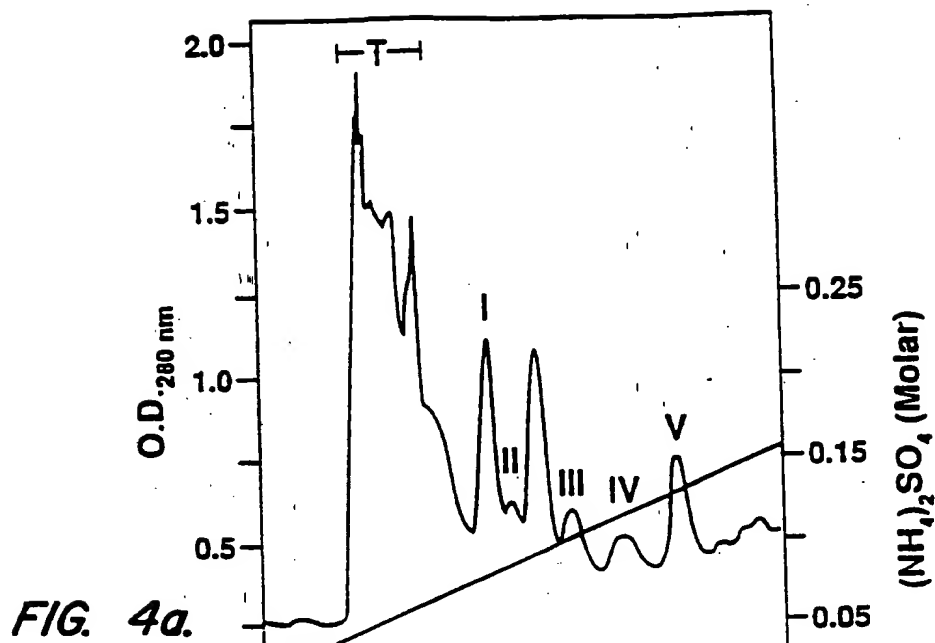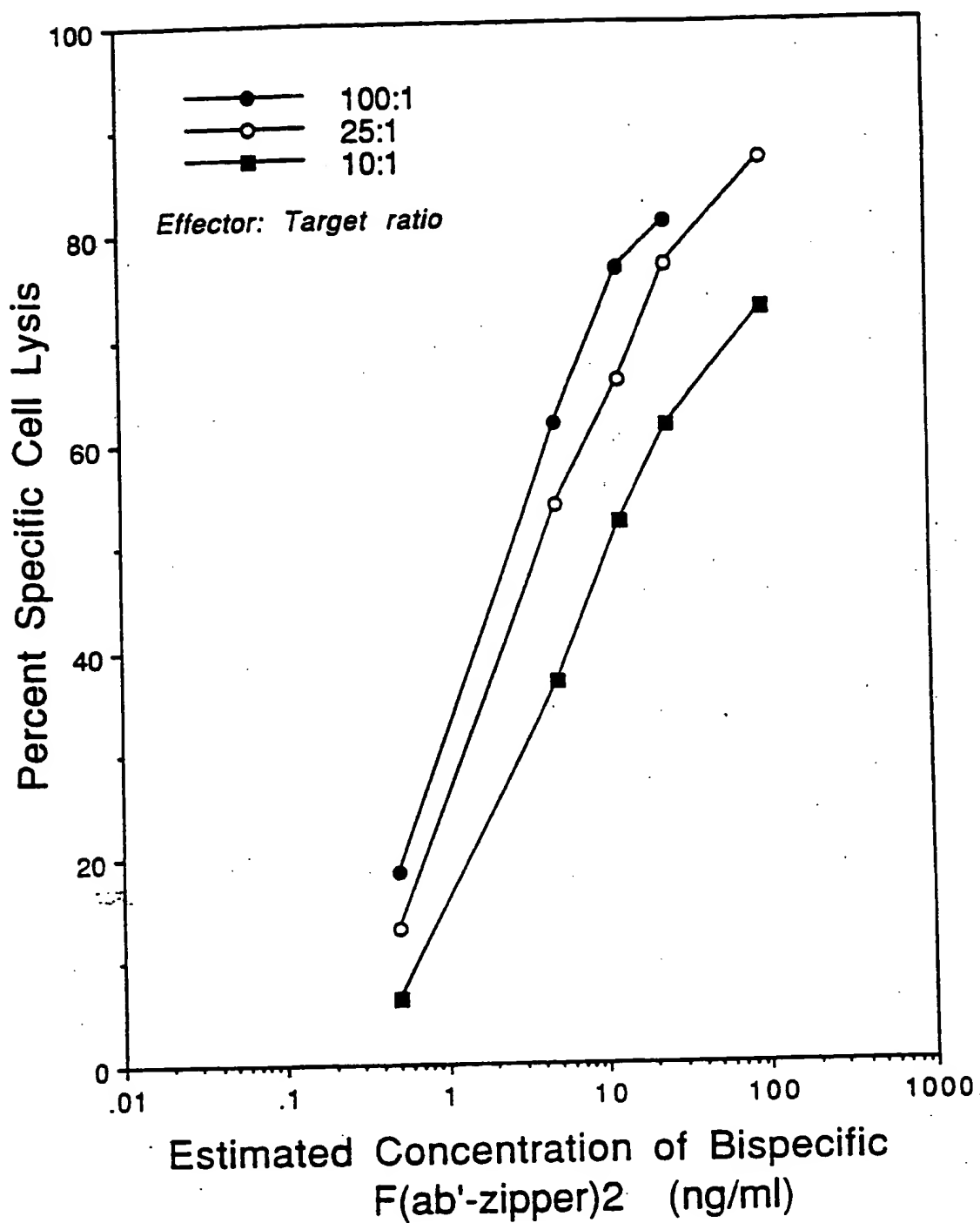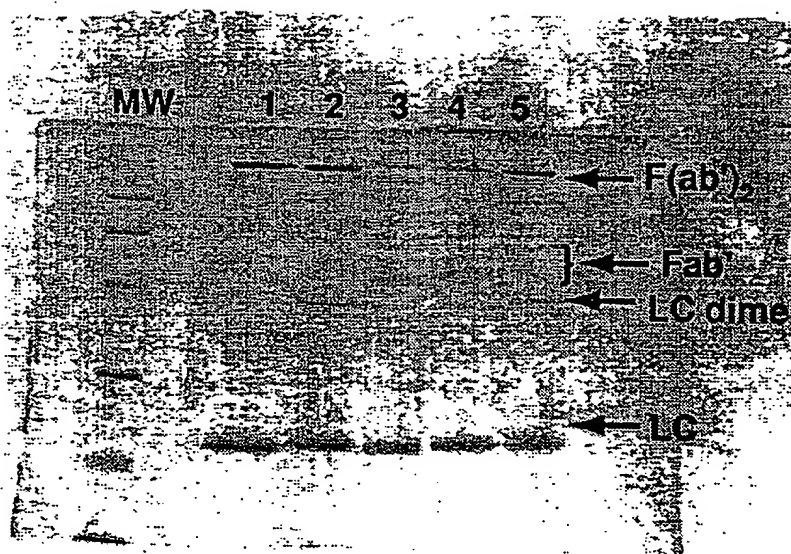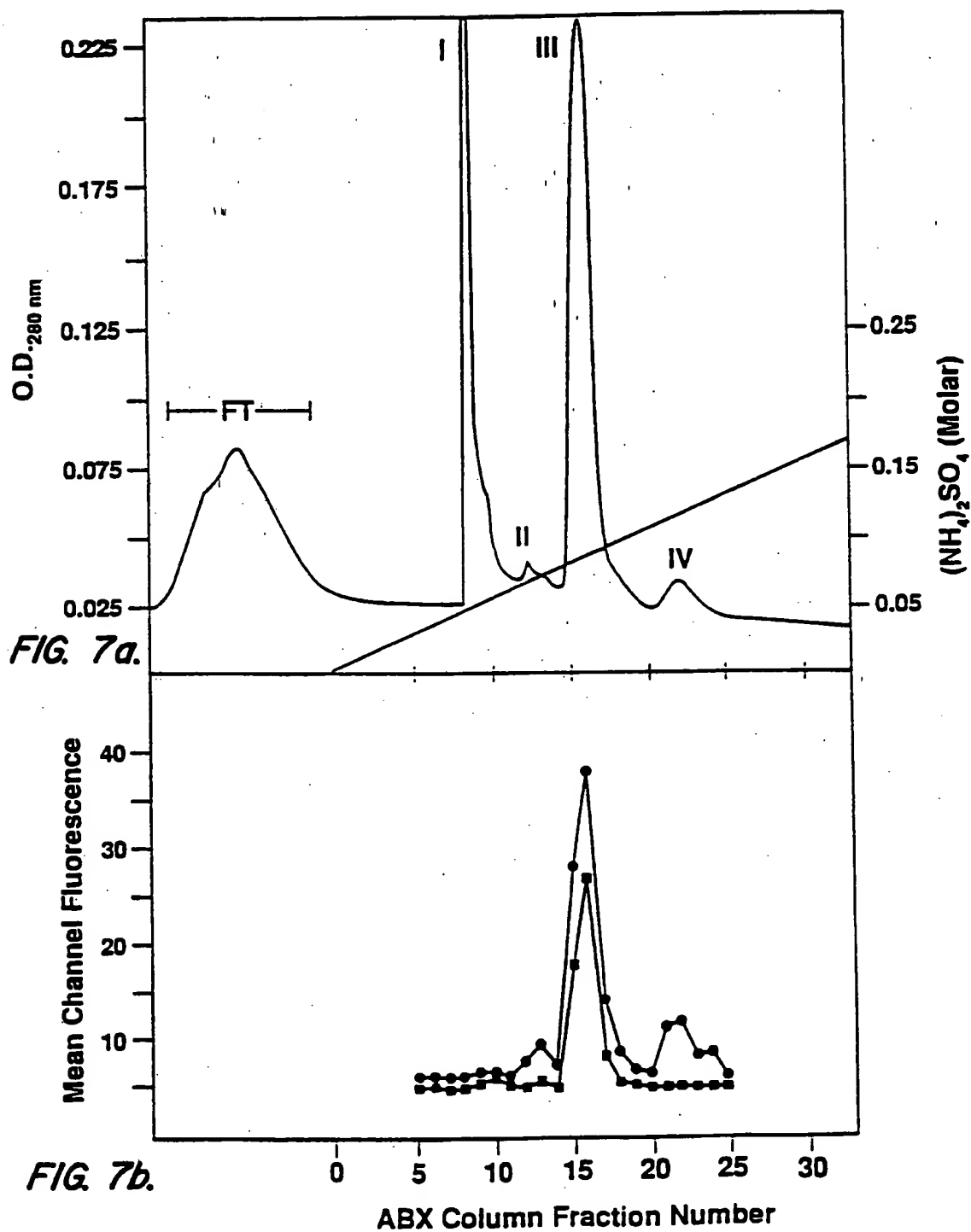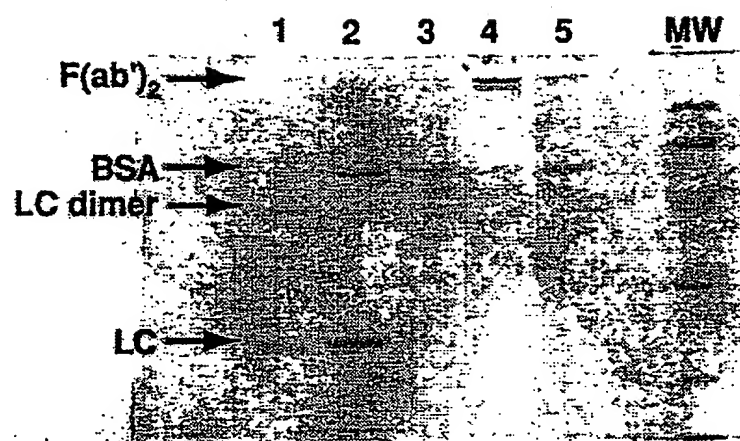
FIG. 8a.
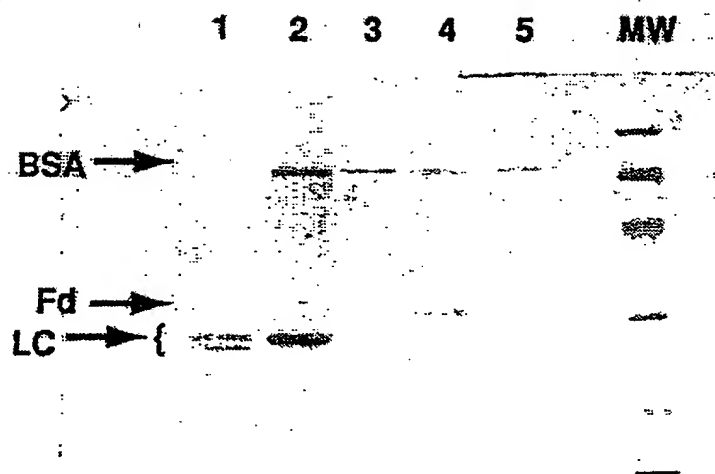
FIG. 8b.
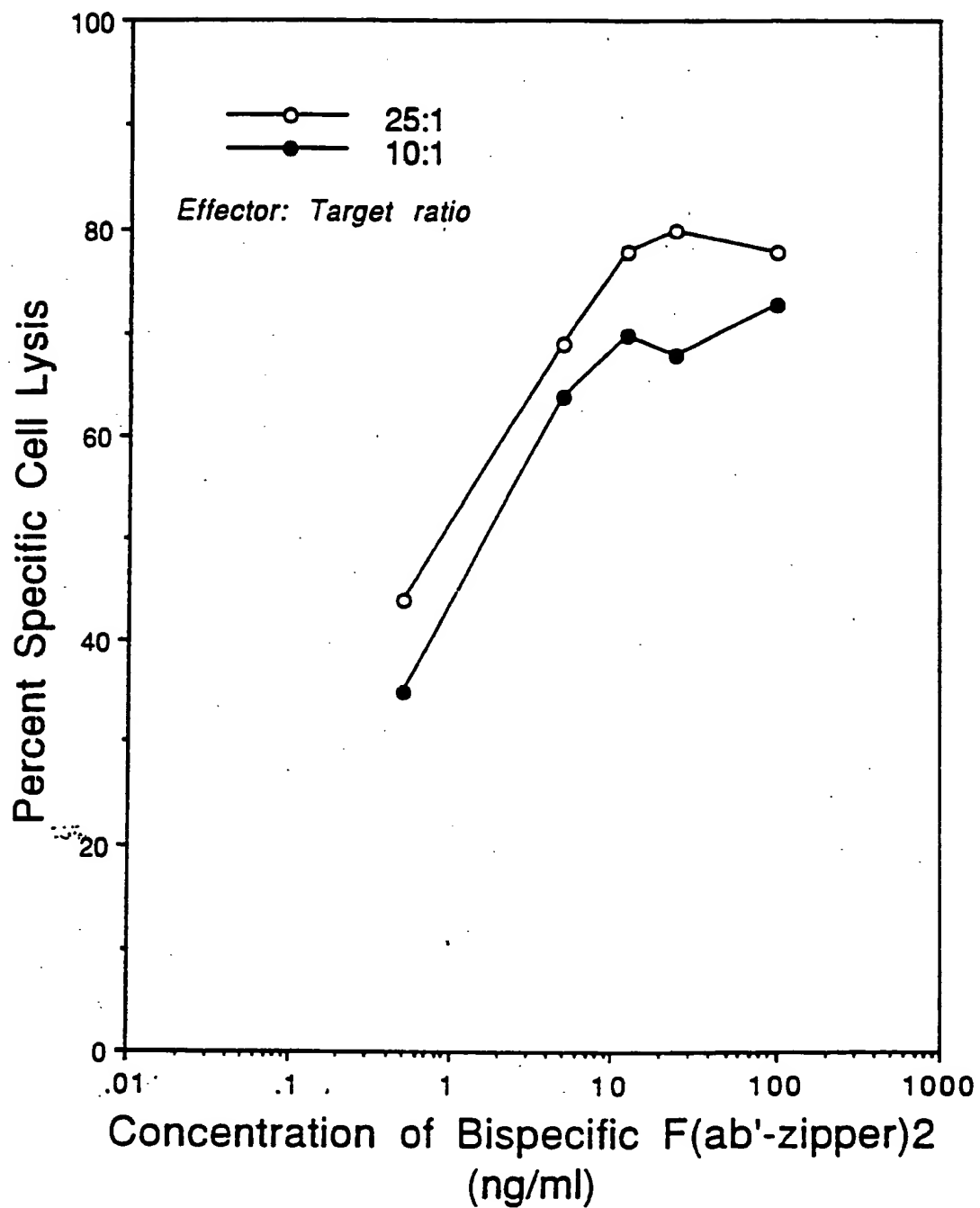
FIGURE 9

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C07K 15/28; C12P 21/06; C07H 15/00; A01N 43/04

US CL   :530/387.3, 387.1; 435/69.6; 536/27; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   530/387.3, 387.1; 435/69.6; 536/27; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, MEDLINE, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Immunology, Volume 139, Number 7, issued 01 October 1987, Glennie et. al., "Preparation and performance of bispecific F(ab'gamma)2 antibody containing thioether linked Fab'gamma fragments", pages 2367-2375, see entire article.	1-25
Y	Science, Volume 245, issued 11 August 1989, O'Shea et. al., "Preferential heterodimer formation by isolated leucine zippers from Fos and Jun", pages 646-648, see entire article.	1-25
Y	Protein Engineering, Volume 4, No. 4, issued 1991, Blondel et. al., "Engineering the quaternary structure of an exported protein with a leucine zipper", pages 457-461, see entire article.	1-25
Y	Proceedings of the National Academy of Sciences, Volume 85, issued October 1988, Gilliland et. al., "Universal bispecific antibody for targeting tumor cells for destruction oby cytotoxic T Cells", pages, 7719-7723, see entire article.	1-25

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 1993	26 FEB 1993

Name and mailing address of the ISA/	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	LILA FEISEE
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*